Biogas Production and Nutrient Recovery from Waste Streams

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Yulin Ye

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Dedication

This thesis is dedicated to my father Danyang Ye and mother Xiangping Zhang.

Abstract

Waste streams such as municipal wastewater and animal manure contain organic materials and nutrients that can be converted and recovered for bioenergy and renewable fertilizer production. In the first part of this thesis, the anaerobic co-digestion of dairy manure with kitchen waste and chicken fat was studied for the purpose of increasing biogas production. The methane yields of co-digestion substrates mixed at different ratios were determined by bio-methane potential tests. The highest methane yield, which was 114% higher than the baseline, was observed when dairy manure was mixed with kitchen waste and chicken fat at the ratio of 1:2:2 (volatile solids based). The mixed substrates were then fed to a lab-scale continuous stirred-tank reactor. The co-digestion was stable and biogas production was 1559 ± 195 mL biogas/L day at organic loading rate as high as 6.8g COD/L day. In the second part, a new approach was proposed for phosphorus removal and recovery from wastewater. Nine strains were identified to have the capability of high phosphorus removal and storage comparable to Polyphosphate Accumulating Organisms (PAOs) in the Enhanced Biological Phosphorus Removal (EBPR) process. Batch experiment using synthetic wastewater showed that *Mucor circinelloides* can remove ~ 72-82% phosphorus when P to COD ratio was roughly 1:100. The phosphorus recovered from wastewater in the form of polyphosphate-containing fungal biomass could be used as fertilizer, providing a potential alternative to biological nutrient removal and a solution to sustainable agriculture.

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1.1 Introduction

Onsite anaerobic digestion (AD) of animal manure brings many benefits to livestock farms, including odor control, reduced solids, deactivation of pathogens, production of bedding materials, and better nutrient management (Sakar, Yetilmezsoy, and Kocak 2009). Moreover, when the methane-containing biogas produced in this process is burned in internal combustion generators, electricity and heat can be produced that not only can cover the energy consumption of farm operation but also may be sold to public grids, creating revenue for farmers. As a byproduct, the heat in flue gas can be captured using a heat exchanger, satisfying the heating need of maintaining appropriate reactor temperature.

Funded by Minnesota State Legislative Commission on Minnesota Resources (LCMR), an Upflow Anaerobic Sludge Blanket (UASB) reactor was installed on Jer-Lindy Farms (Brooton, MN) with the goal of evaluating the economic feasibility of small farm digesters. The farm suffered economical loss since the installation of this reactor in 2008, due to low biogas production and high maintenance cost of the equipment (Janni and Schmidt 2012).

To find a way of improving biogas production, co-digestion of dairy manure with organic wastes including kitchen waste and chicken fat was proposed. In this study bio-methane

potential (BMP) tests were performed to determine an optimized substrates mixing ratio. In addition, a lab-scale continuous stirred-tank reactor (CSTR) treating a mixture of dairy manure with organic wastes including kitchen waste and chicken fat was prepared and evaluated in terms of biogas production at different organic loading rates.

1.2 Background

1.2.1 AD overview

AD is a natural process that converts organic materials to biogas. It is widely used to decrease the amount of organic matter. Compared to the traditional energy consuming aerobic treatment, AD has more advantages since it is suitable to most types of organic wastes such as animal manure, waste paper, grass clippings, municipal waste, food and fruit/vegetable processing waste (Yan Liu, Miller, and Safferman 2009). AD provides benefits including odor reduction, production of a renewable energy source (biogas), pathogen and weeds reduction, sludge volume reduction, and enhanced nutrient management (Vandevivere 1999; Wilkie and Ph 2005).

As shown in, the overall conversion process of the complex organic matter into methane and carbon dioxide can be divided into four steps (Gujer and Zehnder 1983): hydrolysis, acidogenesis, acetogenesis and methanogenesis. In the hydrolysis process, macro molecules like proteins, polysaccharides and fats are hydrolyzed by microbial activity into smaller molecules, such as peptides, saccharides and fatty acids. These small molecules are further fermented by another group of bacteria, generating light-weight

volatile fatty acids (VFAs) in a process called acidogenesis. If the reaction is complete, all intermediate products should be converted into acetate, hydrogen and CO_2. In the final step acetate, hydrogen and CO_2 are converted to CH_4 by two groups of methanogenic archaea, i.e. Aceticlastic methanogens and hydrogen oxidizing archaea. Aceticlastic methanogens split acetate into CH_4 and CO_2. Hydrogen oxidizing archaea can utilize hydrogen as an electron donor and CO_2 as an electron acceptor to produce methane (Appels et al. 2008).

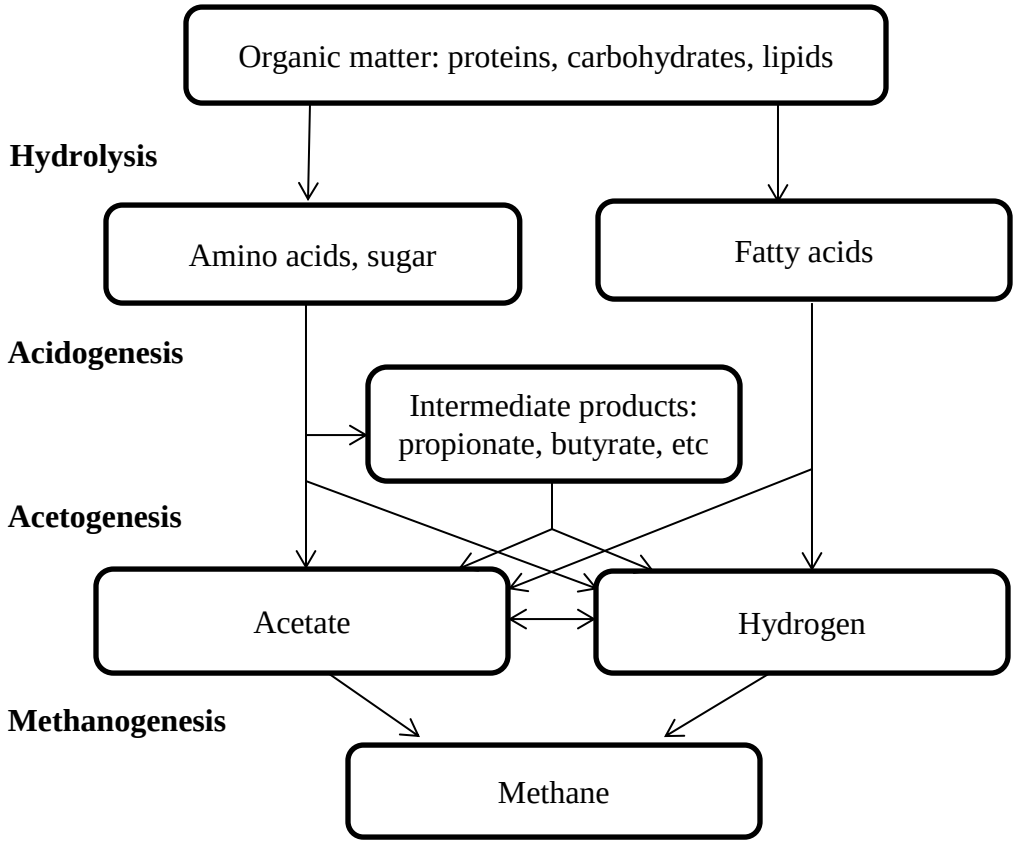


Figure 1 Schematic of material conversions in anaerobic digestion

Since bacteria can get relatively small energy from fermentative catabolism, the yield coefficient, which is the ratio of biomass production to feeding substrates, is much lower

than in aerobic processes. As a result, a large fraction of the digested organic matter (85%-95%) is converted into biogas (van Haandel and van der Lubbe 2012).

Important operation parameters of AD include temperature, pH, hydraulic retention time (HRT), solid retention time(SRT) and organic loading rate (OLR), etc.

Temperature

Three temperature ranges are commonly used in anaerobic conditions; psychrophilic (5-20 ℃), mesophilic (30-35 ℃) and thermophilic (50-60 ℃) (Gerardi 2003). Psychrophilic condition are limited in applications due to low microbial activity is less optimal. Thermophilic conditions give a faster bacterial growth and waste degradation, while it does not remove odor as complete as in mesophilic conditions (Burke 2001). On the other hand, it requires high energy input to maintain operational temperature. Mesophilic conditions are most popular in full-scale applications, because anaerobic mesophiles exists in natural substrates such as manure, and its relatively low energy input makes it more economic feasible.

pH

Methanogen are a group of bacteria that grow extremely slow and very sensitive to pH changes (Khanal 2011). The optimal pH range of methane production is neutral to slightly alkaline, from 6.3 to 7.8 (Leitão et al. 2006). If the acids production rate exceeds its utilization rate, which is typically caused by organic overload or biomass wash-out,

acids will build up in the reactor and lower the pH. When the system is acidified, the activity of methanogen will be inhibited.

Hydraulic retention time (HRT) and Solid retention time (SRT)

HRT is the averages time that feeding material stay in a reactor. It equals the effective reactor volume divided by the feeding flow rate (HRT=V/Q). The hydraulic retention time is an important operational parameter because it controls the contact time available for biomass and substrate and thus determines the degree of treatment.

SRT is the average time that solids spend in the reactor. Since biomass is a part of solids in the reactor, SRT also determines the available time for biomass growth. In a reactor where biomass is not retained or recycled (i.e. a CSTR), HRT equals SRT. However some reactor designs such as upflow anaerobic sludge blanket reactors, biofilm reactors and membrane reactors can decouple HRT and SRT by separating solids liquid flow and solids flow (Khanal 2011). Biomass in these reactors can reach higher concentrations, allowing high treatment capacity with relatively small reactor volume and lower HRT.

Organic loading rate (OLR)

OLR is the amount of organics measured as Chemical Oxygen Demand (COD) or Volatile solids (VS) loaded per unit volume of reactor per day (g COD or VS/L day). High OLR increases volumetric biogas production but it could risk the stability of AD system when the loading exceeds treatment capacity of the reactor, causing acids

accumulation and inhibition (Borja, Banks, and Wang 1995). Moreover, when a reactor is over loaded, the digestion cannot be completed, resulting in deceased methane production efficiency. Generally speaking, reactor types that retain or recycle biomass allow higher OLR because of their high metabolic rate.

As discussed above, reactor design has significant impacts on HRT, SRT and OLR of the AD process (see Table 1). The selection of appropriate reactor type and configuration is essential to maximize bioenergy production (Khanal 2011). Typical types of anaerobic digesters that are widely used in manure waste treatment include covered lagoon, completely mixed reactor, Upflow Anaerobic Sludge Blanket (UASB) reactor and Plug Flow Reactor (PFR) (Burke 2001). Their diagrams are shown in Figure 2.

Table 1 Typical types of anaerobic digesters in manure waste treatment ("Dairy Manure Anaerobic Digester Feasibility Study Report" 2009)

	Covered lagoon	**PFR (slow rate)**	**CSTR**	**UASB**
Operating temperature	Psychrophilic	Psychrophilic	Mesophilic or thermophilic	Mesophilic
Foot print	Large	Large	Medium	Small
OLR	Low	Low	Medium	High
HRT	>48 days	20-40 days	20-30 days	less than 10 days
Biogas yield	Low	low	High	High
Cost	Low	low	Medium	High

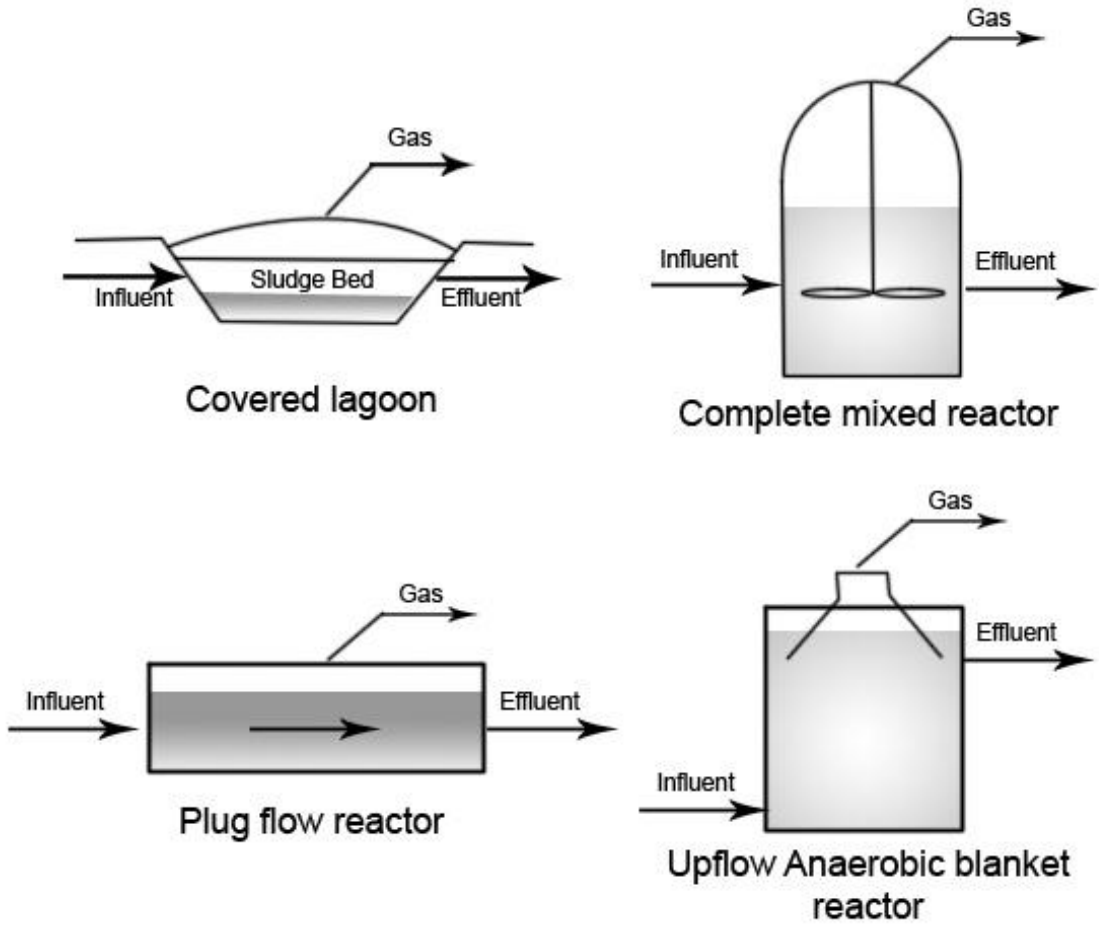


Figure 2 Diagrams of common AD reactors

Covered Lagoon

Covered lagoon is one the simplest forms of anaerobic digester. Without mixing, biomass tends to settle and form a sludge bed, reducing the contact between microbial consortia and bulk liquid. The temperature is not controlled by this type of reactors and it is usually run at psychrophilic condition, resulting in low reaction rate and poor biogas conversion efficiency (Burke 2001).

Plug flow Reactor (PFR), slow rate

PFR is another simple type of reactor that is used to treat manure with relatively low solids content. In a plug flow reactor the waste enters on one side of the reactor and exits on the other. In animal manure treatment applications, a PFR is built partially or fully below grade to limit the demand for supplemental heat. Since the plug flow digester is a growth based system, it is less efficient than a retained biomass system (Burke 2001).

Continuous Stirring Tank Reactor (CSTR)

A CSTR, also called a completely mixed reactor, is an enclosed and heated tank with a mechanical, hydraulic, or gas mixing system (EPA 2013). CSTR is a proven technology that achieves reasonable conversion from solid to gas (Burke 2001). One major disadvantage of CSTR is that biomass is washed out with effluent and can't be retained in the reactor. Thus its SRT usually equals HRT, requiring relatively longer retention time or larger reactor volume to achieve the same degrees of treatment compared to retained biomass systems.

UASB reactor

An Upflow Anaerobic Sludge Blanket (UASB) reactor is a suspended growth system where the hydraulic condition is designed to selectively retain dense biomass aggregates known as granules (Khanal 2011). By maintaining a superficial upflow velocity at 1-6m/h, granules with superior settling characteristics are induced and they form a sludge

blanket on the bottom of the reactor that is high in biomass concentration. Thus, UASB can operate with a SRT as long as 200 days and a HRT as low as 6h (Hulshoff Pol et al. 2004). This type of reactor is suitable for the treatment of high-strength wastewater streams with relatively low solids content.

1.2.2 Co-digestion of dairy manure and organic wastes

A balanced nutrient supply and a stable pH are prerequisites for reliable AD process performance. The optimized C/N ratio of 30:1-20:1 is recommended by EPA's AgSTAR program for better gas yields (AgSTAR 2012), as well as other nutritional factors such as phosphorus, trace metals etc. Henze et al summarized in a literature review (Henze and Harremoës 1983) that the minimum COD:N:P ratio for anaerobic system is 100:2:0.3. Co-digesting organic enriched wastes with manure can significantly increase the biogas production since they bring nutrients balance for the anaerobic digestion. For many types of organic wastes e.g. high-fat content wastes, fruit and food waste, the carbon content is significantly high comparing to the nitrogen, phosphorus and trace nutrients (Yan Liu, Miller, and Safferman 2009), making them good sources of biodegradable organics for co-digestion. Manure is also one of the best co-digestion materials due to its abundance in indigenous anaerobic bacteria and its high alkalinity, which increases digester resistance to acidification (Sosnowski, Wieczorek, and Ledakowicz 2003).

1.2.3 The UASB digester at Jer-Lindy farm

Jer-Lindy Farm near Brooten (MN, USA) is a small dairy farm with less than 200 cows. Designed and constructed by Andigen LLC. Logan Utah, an UASB type anaerobic

digester was installed in 2008 (Figure 3). The project was funded by LCCMR and was initiated to study the economic feasibility of running anaerobic digester on dairy farms of its scale. The AD system includes a 33000 gallon reactor, an external bypass tube heat exchanger, a 40 kW engine generator set, a system of controls and monitoring equipment, and a drum screen solids separator. The generator was designed to produce about 430 kWh/d.

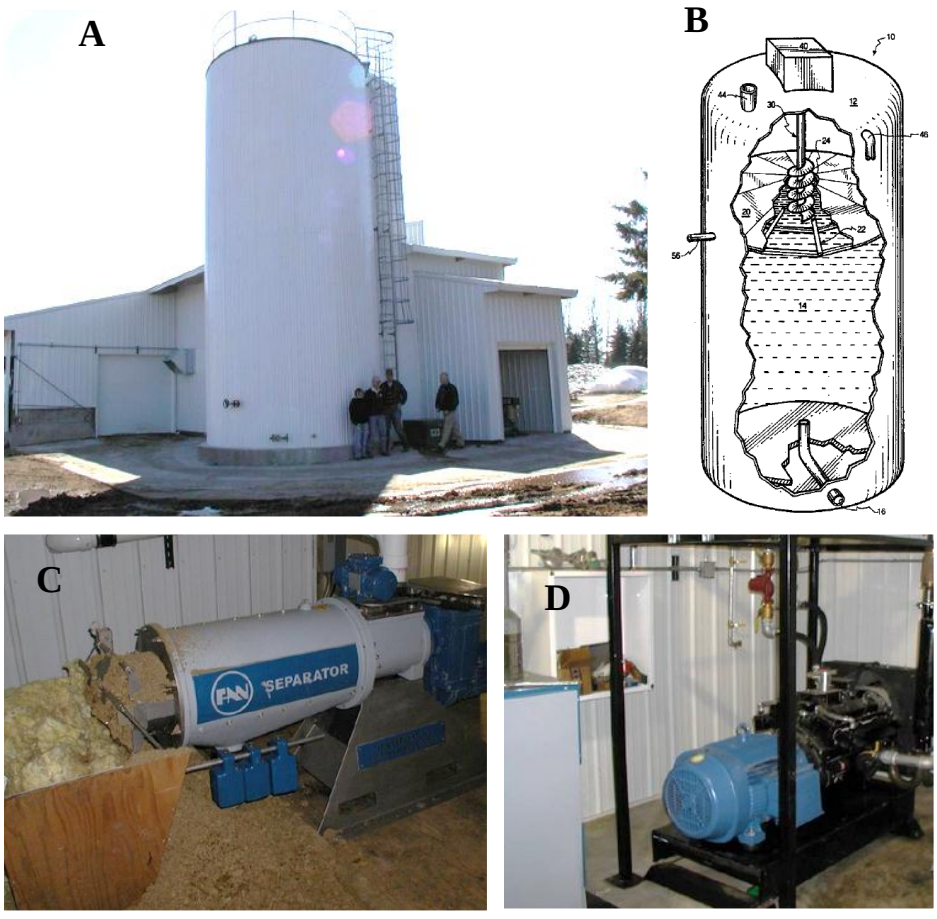


Figure 3 The anaerobic digester at Jer-Lindy farm

(A) reactor view from outside; (B) reactor design (Hansen and Hansen 2005) (C) drum screen solid separator; (D) internal combustion generator

This UASB reactor has a patented design (Hansen and Hansen 2005) that a septum or other parts are positioned so that the solids are forced toward the lower part of the reactor, as shown in Figure 3 (B). Alternatively, these parts can be positioned to pull up solids when it is desired. This design is claimed to enhance the solids retention, allowing a longer SRT for a more complete treatment of solids-containing streams.

To decrease the impact of solids on equipment maintenance, a drum screen solids separator with pore size of 0.2 mm (Figure 3 (C).) was installed in 2012 to remove solids from raw manure. Although the occurrence of equipment issues decreased significantly since this modification, the biogas production was also reduced by almost a half. Start from 2012, the farm was seeking to increase biogas production through co-digesting manure with off-farm organic wastes.

In this research, the co-digestion of manure with two types of organic wastes (kitchen waste, chicken fat) was studied. A 30-day BMP test was carried out and the digestibility of all three substrates plus 16 combinations of their mixture at different ratios were determined. In addition, a lab-scale CSTR was prepared and the methane production efficiency at two different OLRs was evaluated under mesophilic conditions.

1.3 Materials and methods

1.3.1 Co-digestion materials

Dairy manure was sampled from Jer-Lindy Farm (Brooten, MN). The manure was processed at the farm where large solids were removed by pressing raw manure through a drum screen filter (pore size 0.2 mm). Kitchen waste was collected from cafeteria of a local high school near the farm. Chicken fat was sampled from a chicken processing plant (Golden Plump, MN). It is the grease part of chicken processing wastewater and about 68% of its total solid is grease and oil (Minnesota Valley Testing Laboratory Inc.). The characteristics of these three co-digestion substrates are listed in Table 4.

1.3.2 Bio-methane Potential (BMP) test

BMP tests is a batch experiment aimed at evaluating the potential efficiency of anaerobic process for a specific waste (Owen et al. 1979). The assays were performed according to Angelidaki et al. (Angelidaki et al. 2009). The batch experiments were performed in serum bottles of 0.15 L with a working volume of 0.1 L. Substrates and anaerobic inoculum were added to each bottle. The substrate concentration based on volatile solids content (VS) was 4 g VS/L. Substrate mixtures were prepared by mixing screened dairy manure, kitchen waste and chicken fat at different ratios (Table 2)

Table 2 Co-digestion substrate mixing ratio (VS basis)

Treatment No.	VS contribution, %		
	kitchen waste	chicken fat	screened manure
1	0%	0%	100%
2	0%	10%	90%
3	0%	20%	80%
4	0%	40%	60%
5	10%	0%	90%
6	10%	10%	80%
7	10%	20%	70%
8	10%	40%	50%
9	20%	0%	80%
10	20%	10%	70%
11	20%	20%	60%
12	20%	40%	40%
13	40%	0%	60%
14	40%	10%	50%
15	40%	20%	40%
16	40%	40%	20%
17	100%	0%	0%
18	0%	100%	0%
inoculum control	0%	0%	0%

The inoculum to substrate ratio of 2:1 was used based on VS content. The inoculum used in the test came from a full-scale anaerobic digester processing sludge (Blue Lake WWTP, Shakopee, MN). Before incubation the head space of each serum bottle is flushed with nitrogen gas to remove oxygen. The prepared bottles were incubated at 35 ±2 ℃ and continuously shaken at 150 rpm. The experiments were conducted in triplicate for 30 days. The amount of biogas produced was collected by water displacement of a solution of hydrochloric acid at pH 2 in a calibrated glass cylinder

manometer (Figure 4). Assays with inoculum alone were used as a control. The methane produced from the inoculum (from the control bottles) was subtracted from the sample assays. The biogas and methane values presented are expressed for standard temperature and pressure (STP) conditions (0 ℃, 1 atm).

Figure 4 Incubator for BMP test and the glass cylinder manometer

1.3.3 Lab-scale anaerobic CSTR

A continuous stirring tank reactor (CSTR) was set up for the study of organic loading rate (OLR)'s effect on co-digestion methane production. The reactor had a working volume of 1.6 L and was fed with mixed substrates (kitchen waste: chicken fat: screened manure of 1:1:3 VS basis which was equivalent of 45.9g kitchen waste and 73.4g chicken fat per liter of screened manure) every 3-4 days in a fed-batch mode. The mixed substrates was prepared in advance and stored in a -20 ℃ refrigerator. Before Phase I begin, the reactor was inoculated with 10g VS/L sludge from industrial anaerobic digester.

Table 3 Operative conditions and summary of the result obtained in the CSTR reactors

Parameter	Phase I	Phase II
Time (day)	0-13	14-24
Operating temperature (℃)	35±2	35±2
Reactor pH	7.4±0.2	7.7±0.1
OLR (g COD/ L day)	3.4±0	6.8±0.1
HRT (days)	20±0.1	10±0.1

The operative conditions are summarized in Before Phase I begin, the reactor was inoculated with 10g VS/L sludge from industrial anaerobic digester.

Table 3. In Phase I, the reactor was operated at HRT of 20 days and OLR at 3.4 g COD/L day. In Phase II the OLR was doubled by lowering HRT to 10 days. The air-tight reactor was connected to a gas column where the biogas produced was collected by water displacement of a solution of hydrochloric acid at pH 2 (Figure 5). The biogas values presented are expressed for standard temperature and pressure (STP) conditions (0℃, 1 atm). The effluent of reactor was sampled at 3-4 day interval.

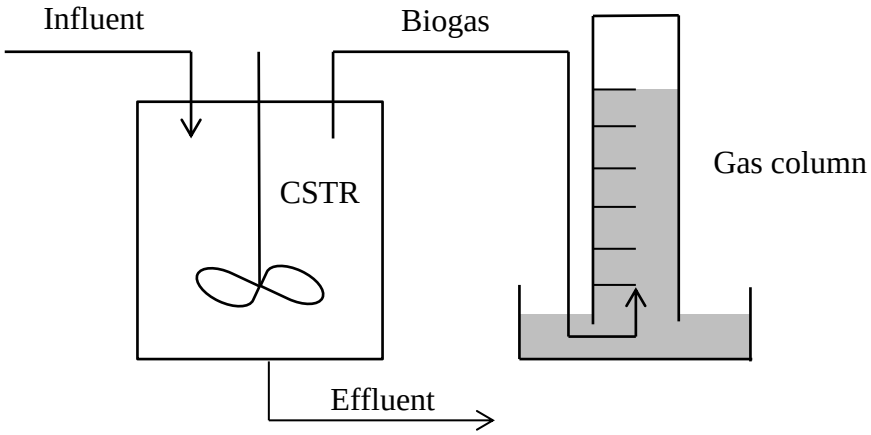


Figure 5 Lab-scale CSTR set-up scheme and photograph.

1.3.4 Analytical methods

Chemical Oxygen Demand (COD), total nitrogen(TN), total ammonium nitrogen (TAN) and total phosphorus(TP) were analyzed by colorimetric methods using commercial testing kits (TNTplus™ 822/827/845, HACH USA) and a UV-Vis spectrophotometer

(Hach® DR 5000™). Soluble COD and soluble phosphorus were measured using the same protocol except that samples are filtrated with 0.45 µm pore size microfiber filter. CH_4 and CO_2 concentrations in the gas samples were measured using VARIAN CP-4900 gas chromatography. Volatile fatty acids (VFAs) were extracted with diethyl ether and the sample was analyzed with an Agilent Tech 7820A gas chromatography. The total VFAs was the sum of concentrations of acetic acid, propionic acid, iso-butiric acid, butyric acid, iso-valeric acid, valeric acid, iso-caproic acid, caprioic acid and heptanoic acid.

1.4 Results and discussion

1.4.1 BMP test

Chemical composition of substrates indicates that (i) Both kitchen waste and chicken fat has high moisture content (i.e. around 70-80%). In addition, they contain high COD concentrations between 25%-50% of their total weight. (ii) Comparing the minimum COD:N:P ratio of 100:2:0.3 which is required for adequate anaerobic digestion, screened manure has an excess of N and P, kitchen waste and chicken fat have a deficiency of N and P respectively. From the chemical composition point of view, it is possible to co-digest these substrates.

Table 4 Characteristics of co-digestion substrates

Parameter	Screened manure (*n*=3)	Kitchen waste (*n*=3)	Chicken fat (*n*=3)
Total solids, TS (g/L, %) w/w	30.9 ±1.5g/L	26.6 ±0.3 %	18.0 ±1.0 %
Volatile solids, VS (%-TS)	72.9 ±0.01 %	95.9 ±0.2 %	88.6 ±0.7 %
Ash (%-TS)	26.1 ±0.01 %	4.1 ±0.2 %	11.4 ±0.7 %
Chemical oxygen demand, COD	39.5 ±0.2 g/L	258 ±10 mg/g	400 ±119 mg/g
Total nitrogen, TN	3.3 ±0.3 g/L	3.4 ±0.1 mg/g	11.6 ±6.3 mg/g
Total phosphorous, TP	0.6 ±0.01 g/L	0.7 ±0.1 mg/g	0.5 ±0.1 mg/g
pH	7.7 ±0.3	-	-
COD:N:P ratio	100:8.4:1.5	100:1.3:0.3	100:2.9:0.1

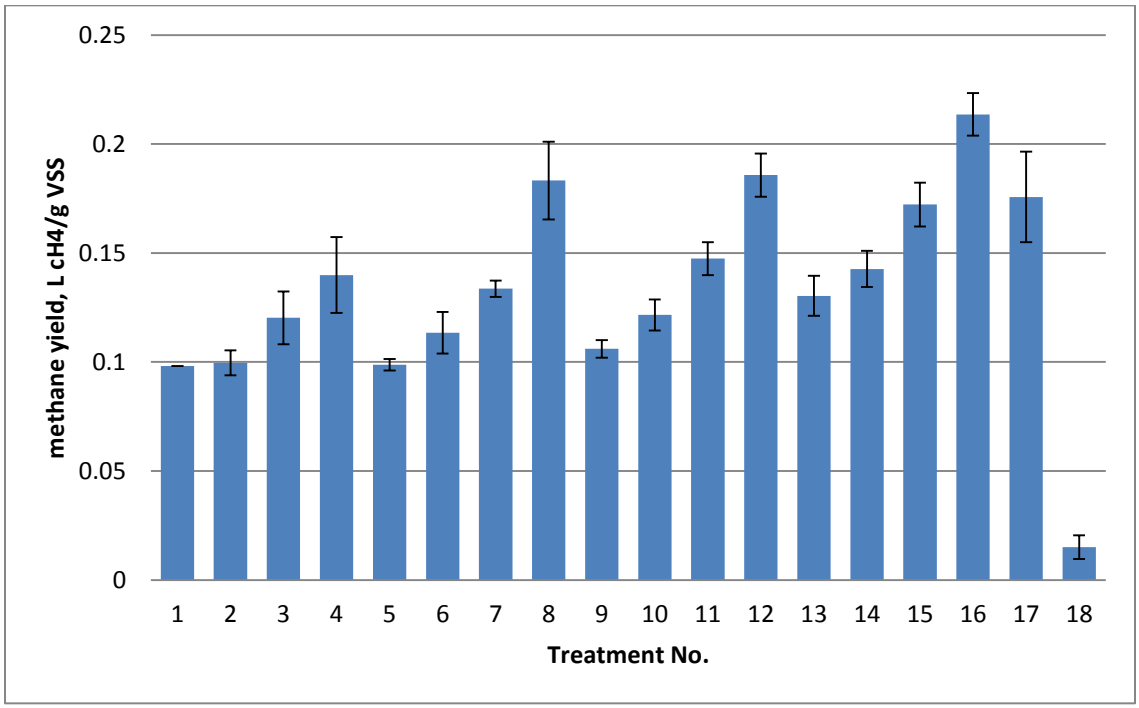


Figure 6 Cumulative methane yield at Day 30

The lowest cumulative methane yield is from chicken fat alone (Treatment 18) i.e. 0.015 L CH_4/g VS. As the final pH of effluent (Table 3) is in the optimal range (7.04-7.28), acidification is not likely to be responsible for the low methane yield. The possibility could be the nutrient in this substrate was unbalanced with high COD and extremely low P (COD:P=100:0.1). It is also possible that chicken fat was lacking in other micronutrient which has not been identified in this test. Among treatments that use single substrate alone, kitchen waste (Treatment 17 which had 0% from screened manure, 0% VS from chicken fat and 100% VS from kitchen waste) gives the best methane yield, i.e. 0.18 ± 0.02 L CH_4/g VS. Although kitchen waste has lower nitrogen content, its biodegradability seems higher than screened manure alone. The methane yield of screened manure was 0.098 ± 0.005 L CH_4/g VS, which was in the same range as the methane yield of the full scale UASB on Jer-Lindy Farm , i.e. 0.04-0.15 L CH_4/g VS (Janni and Schmidt 2012), confirming that the low biogas production was not a problem of conversion process but a problem of the feeding material (screened manure) itself.

In the treatments with mixed substrates (Treatment 2-16), there is a trend that higher methane yields can be achieved by increasing the fractions of kitchen waste and chicken fat in total VS. Treatment 16 which contains around 40% VS from kitchen waste and 40% VS from chicken fat showed the best performance, i.e. 0.21 ± 0.01 L CH_4/g VS, which is 114% higher than using screened dairy manure as single substrate. This mixing ratio was equivalent of mixing 274g kitchen waste and 440g chicken fat with 1 L screened manure. Although the peak of methane yield was not reached due to the high ends of co-digestion substrates fractions in the experiment design were not high enough

to cause a decline in methane yield, it can be concluded that within the range of 0-40% (VS basis), adding both kitchen wastes and chicken fat can improve the overall biodegradability and conversion efficiency of the feeding material. A linear model can be used to describe the relationship between methane yield in mL CH_4/g VS (Y) and the fraction of VS from kitchen waste (x_1) and chicken fat(x_2) in total VS:

$$Y = 123.25x_1 + 193.44x_2 + 91.82, R^2=0.922$$

Similar results were reported in another study (Li, Chen, and Li 2009). As a baseline, the methane yield using cow manure as the only substrate was 0.067 L CH_4/g VS. By mixing kitchen waste with cow manure at the ratio of 1:1 and 2:1 (VS basis), Li et al (2009) observed increases in methane yield, i.e. 0.159 L CH_4/g VS and 0.194 L CH_4/g VS respectively. These values are comparable to our results, indicating that manure is a substrate with low biogas yields and co-digestion with other substrates can increase the methane output.

It should be point out that the high solid content in kitchen waste and chicken fat (26.6% and 18%, respectively) might be a problem for the utilization in UASB-type reactors, because (i) the relatively short HRT of UASB leave the solid content degraded incompletely, (ii) in UASB reactors, a high solid content can decreased the granulation of the sludge bed and could originate anaerobic biomass washout.

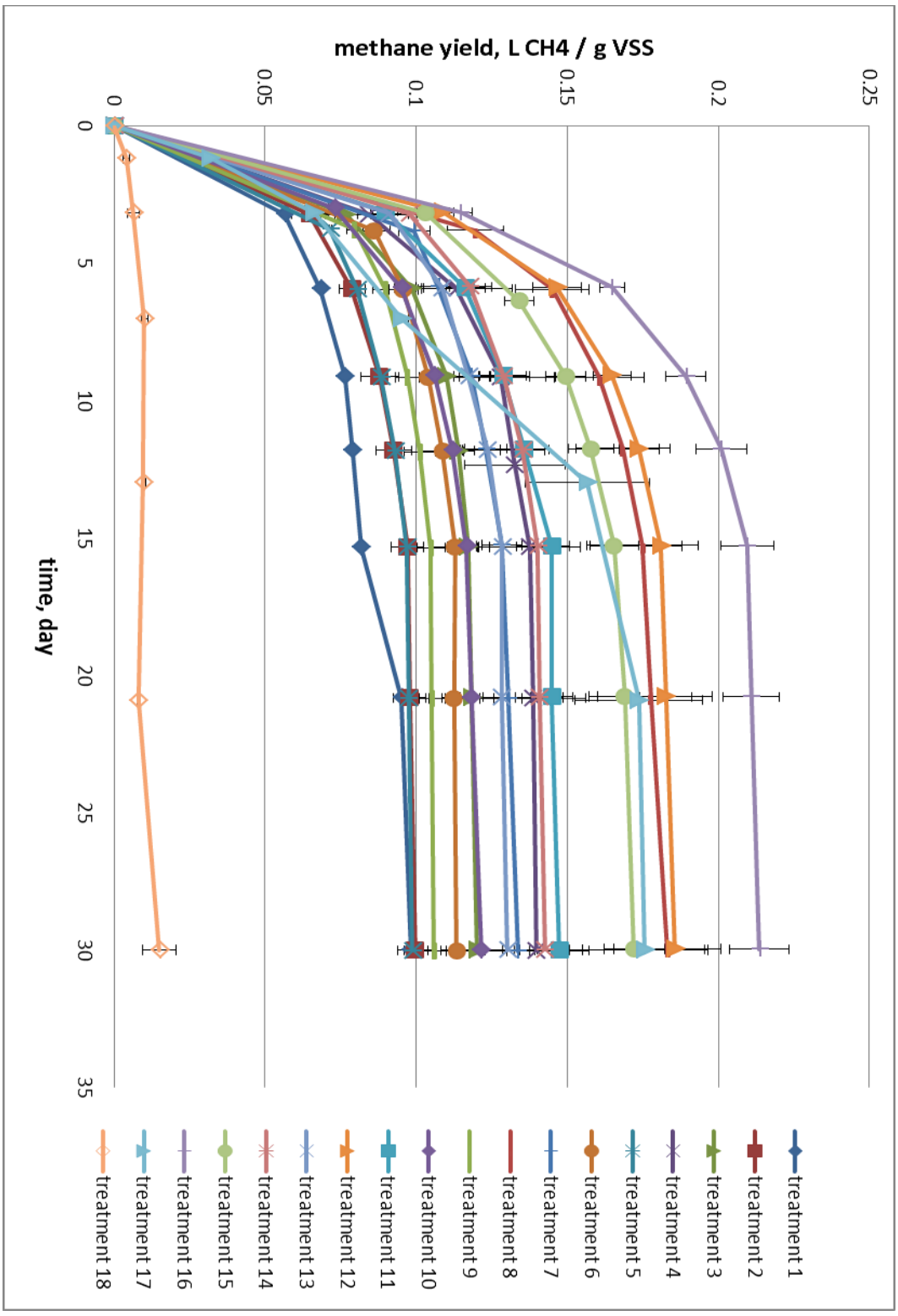


Figure 7 Cumulative methane yield during BMP test

1.4.2 CSTR test at different OLRs

Since the reactor was in start-up period, a conservative feeding strategy was applied: 20% kitchen waste and 20% chicken fat and 60% manure (VS basis) which is equivalent to Treatment 11 in the BMP test was chosen as mixing strategy. The total suspended solids of this mixture was 25.7 ±0.3 g/L and the methane yield was 0.15 ± 0.009 L CH_4/g VS in the BMP tests, 53% higher than digesting dairy manure alone.

Since there was a stabilization period in each phase, the OLR and methane production rate of Phase I and Phase II are calculated from the average of last five days of each phase, and their values are displayed in Before Phase I begin, the reactor was inoculated with 10g VS/L sludge from industrial anaerobic digester.

Table 3 and plotted in Figure 8. The HRT in Phase I was 20 days which is typical for mesophilic CSTR digester (Burke 2001). The OLR under this HRT was 3.4 g COD/ L day and the biogas production was 1072 ±310 mL biogas/L day which corresponded to a conversion efficiency of 63 ±18 % (COD based; 350 mL CH_4/ g COD). After OLR was increased to 6.8 g COD/L day, the biogas production increased to 1559 ±195 mL biogas/L day, which corresponded to a conversion efficiency of 45 ±6 %. The degradation of the conversion efficiency in Phase II indicates that the reaction was not as complete as in Phase I, possibly due to the shortened HRT. The effluent pH in both phases were stable and in optimal range (7.4 ±0.2 and 7.7 ±0.1 respectively). The increase of the OLR seemed not cause acid accumulation in the reactor and allows a continuous production of biogas. This was confirmed by the low concentration of total VFAs (<1000 mg/L) during

both phases (Table 5). The biogas composition was constant in both phases, averaging 61 ±1 % of methane and 39 ±1 % of CO_2.

Yamashiro et al. (2013) reported a methane production rate of 0.53 ± 0.08 CH_4 L / L day digesting dairy manure as single substrate in a CSTR under mesophilic condition (35 ℃). The reactor was operated at organic loading rate of 3.05 g VS/L day (roughly equals to 3.47 g/L COD based on COD/VS of 1.14 for dairy manure) and HRT of 20 days. When co-digestion substrates, i.e. high strength food processing waste was mixed with manure under the same HRT (20 days), the OLR increased to 8.25 g VS/L day (roughly equals to 9.4 g/L COD). As a result, inhibition occurred and biogas production ceased after 13 days of co-digestion (Yamashiro et al. 2013). The inhibition was characterized by total VFA accumulation and pH drop. The author indicated that organic overload and biomass-washout could be responsible for the inhibition.

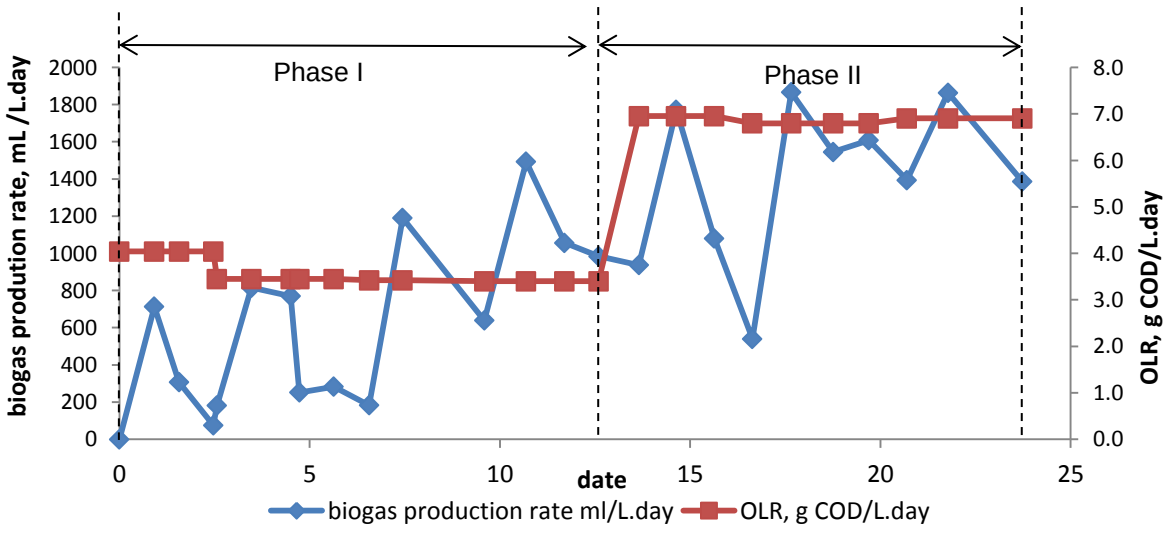


Figure 8 Organic loading rate and biogas production rate

Table 5 shows the characteristics of co-digestion influent and effluents. COD and VS are effectively removed through the AD process, around 56% and 53% removal efficiency was observed in Phase I and 61% and 38% in Phase II. Theoretically, the amount of total nitrogen(TN) and total phosphorus(TP) removed from AD process through biological assimilation is small (Wahal 2010), due to the low growth rate of anaerobic bacteria and archaea. The large differences in TN and TP between feeding material and Phase I effluent could come from the effect of pre-steady-state dilution. In Phase II, TN and TP removal were approximately 20% and 12%, while total ammonium nitrogen increased 13% due to mineralization.

Table 5 Characteristics of CSTR feeding and effluents

	Feeding	Effluent of Phase I	Effluent of Phase II
TS (g/L)	37.8±0.6	18.6±0.5	25.7±0.5
VS (g/L)	30.7±0.2	14.2±0.2	19.1±0.2
TSS (g/L)	25.7±0.3	13.1±1.2	16.7±0.8
VSS (g/L)	22.4±0.6	10.7±0.9	13.2±0.7
Soluble COD (g/L)	19.9±0.4	4.5±0.6	9.5±0.9
Total COD (g/L)	68.8±4.3	30.5±3.9	26.8±0.8
Total nitrogen (mg/L)	3108±459	1795±573	2485±332
Total phosphorus (mg/L)	315±20	194±38	278±17
Total ammonium nitrogen (mg/L)	1137±95	792±217	1310±177
total VFAs (mg CH3COOH/L)	2129±82	403±21	889±3

1.5 Conclusions

In this study, the digestibility of kitchen waste and chicken fat was assessed as substrates for co-digestion with dairy manure. The BMP tests showed that the co-digestion of dairy manure with kitchen waste and chicken fat could improve the methane yield and balance the nutrient composition of the substrates. When the fractions of kitchen waste and chicken fat were within the range of 0-40% (VS basis), significantly improvement on the methane yield was observed (up to 114%). The data can be fitted into a multivariate linear model with an R^2 of 0.922. The highest methane yield was observed at a mixing ratio of 2:2:1 (kitchen waste : chicken fat : screened manure) achieving values of 0.21 L CH_4/g VS. A mixture of kitchen waste, chicken fat and screened dairy manure of 1:1:3 was fed to a CSTR operated at maximum OLR of 6.8 g COD/ L day and at a HRT of 10 days under mesophilic conditions. The conversion efficiency of the CSTR decreased in Phase II due to the shortened HRT.

Chapter 2: Phosphorus accumulating fungi and its potential in wastewater treatment for P removal and recovery

2.1 Introduction

Phosphorus (P) and nitrogen (N) as key cell components of all living organisms are limited nutrients in nature water body. As algae and other autotrophic organisms can utilize inorganic carbons from atmosphere (CO_2), when P and N compounds produced by human activities are discharged into rivers and lakes, the reproduction of these organisms is accelerated, causing environmental nuisance known as algal bloom, which depletes Dissolved oxygen and kills fishes and other organisms in the water (Mall et al. 2002). Therefore massive discharge of P is regulated by pollution control agencies globally.

On the other hand, phosphorus is an important nutrient for crop production. Modern agriculture depends heavily on synthesized fertilizer. Since 1980, the consumption of phosphorus fertilizer is relatively stable at 14 million metric ton P per year (Yi Liu et al. 2008). However phosphate rock - the raw material for phosphorus fertilizer production - is a non-renewable resource. At current excavation rate, the mineral conservation of phosphorus is predicted to be exhausted in 50-100 years (Cordell, Drangert, and White 2009). Economically recovering phosphorus from wastewater provides an ideal solution to address challenges on both protecting surface water from eutrophication and finding a sustainable way of supplying phosphorus fertilizer for food production.

Researchers in the author's lab recently found out that one of our filamentous fungi

strains, *Mucor Circinelloides* (ATCC 1216B) can obsessively accumulate high amount of phosphorus during its cell growth, easily reaching to 5-7% of the dry cell biomass. This is at least compatible to most of the Polyphosphate Accumulating Organisms (PAOs) strains found in wastewater treatment plant. Based on this finding, screening and identification of phosphorus accumulating fungi was carried out. The phosphorus removal pattern was studied in batch experiments using *Mucor Circinelloides* as a benchmark. Massive storage of polyphosphate was identified in its fungal hyphae using staining method. The strain's potential in wastewater treatment was evaluated using flasks culture with synthetic wastewater as media. Finally, the phosphorus removal test was performed using real wastewater, i.e. effluent of sludge dewatering process and dairy manure.

This research proposed a new possibility of P removal and recovery, which is totally different from the currently prevailing Enhanced Biological Phosphorus Removal (EBPR) process. The phosphorus recovered in the form of polyphosphate-containing fungal biomass can potentially be used as fertilizer, providing a sustainable solution to the problem of P fertilizer industry.

2.2 Background

2.2.1 Phosphorus removal technology overview

Since large P-containing particles can be removed through screening and sedimentation, which are considered “primary treatment” in wastewater treatment (Tchobanoglous et al. 2003), the term “phosphorus removal” discussed in this thesis refers to the removal of soluble forms of phosphorus.

Chemical precipitation and biological assimilation are the two major technologies to remove phosphorus from wastewater (de-Bashan and Bashan 2004). Both technologies share the same principle: first converting soluble phosphorus to insoluble forms, such as metal salts, microorganism biomass in activated sludge, or plant tissue in a constructed wetland, then withdrawing these solids from effluent.

Ferric, aluminum, calcium salts are commonly generated in the chemical precipitation method (Frossard, Bauer, and Lothe 1997), then non-soluble phosphate salts (e.g. ferric phosphate, aluminum hydroxide phosphate, calcium phosphate hydroxyapatite and magnesium ammonium phosphate – struvite) are removed by sedimentation in later process. Complete phosphorus removal is achievable by adjusting metal salt dosage according to the concentration of phosphorus in wastewater. Disadvantages of chemical precipitation include increased sludge volume and the cost of chemicals. Most importantly, the bioavailability of these non-soluble salts is poor, making the sludge non-reusable for fertilizer production (de-Bashan and Bashan 2004).

The biological wastewater treatment processes include trickling filters, lagoons, stabilization ponds, constructed wetlands and activated sludge (Gray 2010). In these processes the soluble phosphorus is either uptake by microorganism cells or plant tissues and assimilated into their biomass for growth and reproduction. By separating the biomass from liquid phase, net removal of phosphorus can be realized.

Instead of assimilating phosphorus stoichiometrically for microbial growth, an modified activated sludge process called Enhanced Biological Phosphorus Removal (EBPR) takes the advantage of enhanced storage of phosphorus and "luxury P uptake" by a group of bacteria known as Polyphosphate Accumulating Organisms (PAOs) (Mino, Loosdrecht, and Heijnen 1998). Although EBPR has been widely implemented in wastewater treatment plants, its discovery back to late 1950s was an "accident" (Srinath, Sastry, and Pillai 1959; Seviour, Mino, and Onuki 2003) and the composition of PAOs involved in this process remained unclear for a long period of time. In order to have a better control over the process, studies focused on identification of PAOs were recently carried out using modern technique such as Fluorescence In Situ Hybridization (FISH) (Oehmen et al. 2007). It is shown that bacteria species Betaproteobacteria and Actinobacteria demonstrate significance in P removal (Wagner et al. 1994). Further isolation of PAO has yet to be conclusively achieved.

Different from regular activated sludge systems, EPBR process has an anaerobic reactor prior to regular aeration basins. Figure 9 shows a simple form of EBPR, the A^2/O process. After primary treatment, the influent wastewater go through anaerobic reactors

and anoxic/aerobic reactors in sequence when PAOs uptake P excessively (discussed later). After settling down in the clarifier, a portion of sludge is returned to anaerobic reactor to maintain an appropriate population of microorganisms. The recirculation flow in this diagram is intent for nitrogen removal through circulating nitrate-containing wastewater back to anoxic reactor, where denitrification process takes place (Adrianus van Haandel 2007). Some more complicated modifications of EBPR, such as modified University of Cape Town process and 5-stage Bardenpho process, involve multiple recirculation flows and anaerobic/anoxic/aerobic cycles(Oehmen et al. 2007).

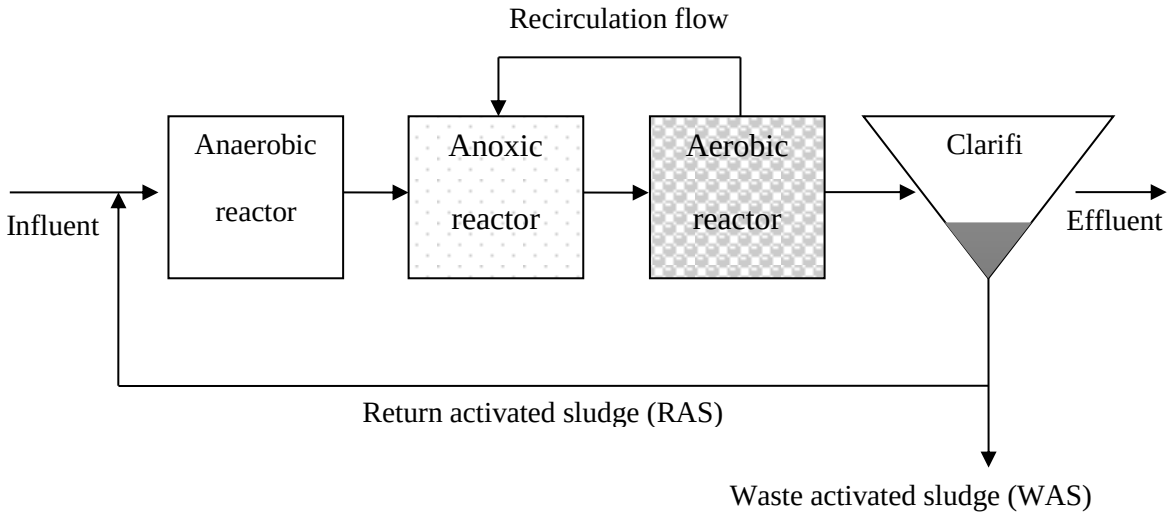


Figure 9 Diagram of EBPR (an A^2/O process)

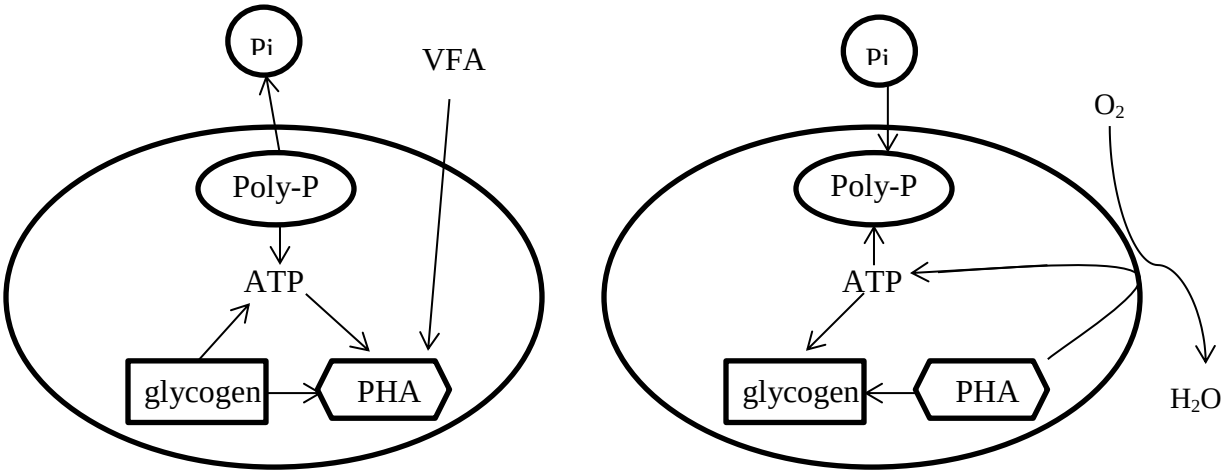


Figure 10 Diagrams of PAO metabolism in anaerobic and aerobic conditions. PHA: polyhydroxyalkanoate, Poly-P: polyphosphate.(Yuan, Pratt, and Batstone 2012)

Under anaerobic conditions, PAOs uptake volatile fatty acids (VFAs) as carbons source from surroundings and synthesize polyhydroxyalkanoate(PHA) as energy storage material. The capability to utilize VFAs as carbon source gives PAOs advantage during anaerobic condition, where PAOs are selected and enriched(Yuan, Pratt, and Batstone 2012). At the same time, poly-phosphate is hydrolyzed to provide ATP under anaerobic conditions, releasing phosphate into environment. In aerobic condition that follows, more phosphorus than the amount that is released during anaerobic phase is uptake by PAOs. Apart from the phosphorus used for regular cell growth, a large amount of phosphorus is stored in the cell as polyphosphate granules, accounting for up to 15% of dry weight(Crocetti et al. 2002). After clarification process, these PAOs biomass end up in

“sludge” or “biosolids”, creating a high-phosphorus solid stream that can be used for land application if its toxic material or heavy metal level is not significant.

2.2.2 Polyphosphate

Figure 11 shows the chemical structure of polyphosphate (poly-P). These linear polymers are composed of 4 to 10000 of phosphate monomers linked by energy-rich phosphoanhydride bonds, and their biosynthesis is catalyzed by Poly-Phosphate Kinase (PPK)(Kornberg 1995).

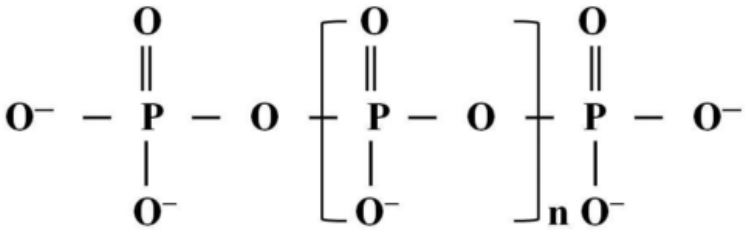


Figure 11 polyphosphate molecule

Based on chain length, two fractions of poly-P are distinguished: acid-soluble and acid-insoluble. Acid soluble poly-P has a short chain of up to about 20 Pi unit, and acid-insoluble poly-P has more than 20 units in its chain(R. E. Beever and D. J. W. Burns 1980).

The primary function of poly-P in microbial cells is serving as energy and phosphate storage(Achbergerova and Nahalka 2011). However some studies have shown that it have other important roles in different organisms. Kornberg and his group summarized the functions of poly-P in a review article(Kornberg 1995), including: A means of storing

energy, A reservoir for phosphate, A chelator of metal ions, A buffer against alkali ions, A channel for DNA entry, and A regulator of stress and survival.

A common method used by wastewater industry to visualize cellular poly-P granules and identify PAOs is Neisser staining (Serafim et al. 2002), where samples on the microscope slides are stained with Methyl Blue, followed by rinsing and counterstaining with Bismarck Brown. Poly-P granules appear dark purple or black and other cell structures are yellow or brown under 1000X optical microscope. In some cases the entire cell is Neisser positive (e.g. *M. phosphovorus* in aerobic EBPR process). This phenomenon can be interpreted as the storage of large amount of poly-P (Serafim et al. 2002).

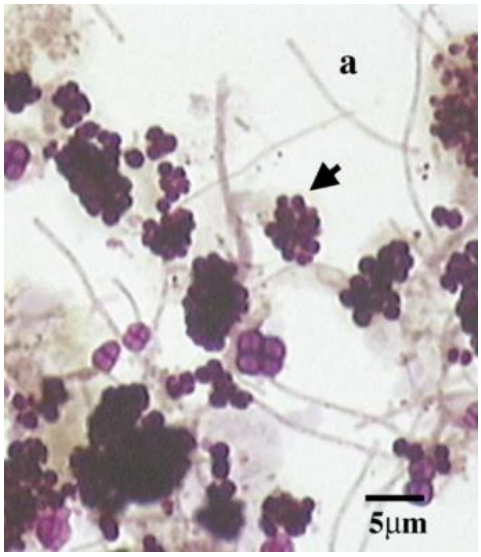


Figure 12 Neisser staining of EBPR sludge (Serafim et al. 2002)

2.2.3 Wastewater treatment using fungi

While many popular biological wastewater treatment technologies including activated sludge process and EBPR are discovered empirically or accidentally (Seviour, Mino, and Onuki 2003), another approach to develop new process is through rational design by selecting specific microorganisms. Microalgae treatment process development is an example of using specific organism(s) in wastewater treatment application (Wilkie and Mulbry 2002; Aslan and Kapdan 2006), owing to algae's ability in nutrient removal and its potential in value-added byproduct recovery. Comparatively, filamentous fungi are not receiving equal attention probably because the term "filamentous" is often related to activated sludge bulking which is considered as process nuisance. In this section fungi's role in sewage plant and some investigations on fungal wastewater treatment is reviewed. Phosphorus accumulating fungi and their potential in P removal and recovery are discussed later in this section.

Filamentous fungi, also known as molds, are higher forms of microorganism, belonging to the domain Eukaryota and the kingdom Fungi. In the ecosystem, filamentous fungi play the role of degrader and cause the decay of organic materials. The extracellular enzymes they produced are capable of degrading recalcitrant substance such as cellulose, lignin, and many types of xenobiotics. Filamentous fungi are also important players in biotechnology and food industry, producing various foods, pharmaceuticals and enzymes.

Around 950 species of fungi are reported to naturally present in domestic wastewater and polluted waters (Sankaran et al. 2010). In municipal wastewater treatment, fungi is a

component of biofilm in moving bed bioreactors (MBBR) and trickling filters (Akpor and Muchie 2010), although the biological process in these reactors are considered to be uncontrolled natural growth of a consortium of microorganisms, and fungi's role is not receiving specific attention. Some studies that specifically using fungi are discussed here.

Fungal remediation has been used to treat toxic streams, for example, textile wastewater containing dyes (Park, Lee, and Park 2005). Fungi have many advantages over bacteria in the treatment of this type of wastewater, including their resistance to inhibitory compounds, lower oxygen and carbon source concentration requirements, and the extracellular enzymes. The ligninolytic enzymes produced by white rot fungi is capable of degrading aromatic compound, including PAH and other recalcitrant compound (Mester and Tien 2000). The low specificity and the extracellular feature of this type of enzyme makes white rot fungi perfect agent bioremediation.

Previous data also revealed that fungi have potential in nutrient removal. In a study using domestic wastewater as media (Thanh NC 1973), *Trichothecium roseum* removed 97.5% of phosphate; *Epicoccum nigrum*, *Geotrichum candidum* and *Trichoderma sp.* removed ammonia (84%), total nitrogen (86.8%) and COD (72.3%), respectively. It was also proposed that that some fungi have both nitrification and denitrification pathways (Guest and Smith 2002).

Typically, fungal cell has a P composition of 100-300 micro mole P/g dry weight (0.31%-0.93% d.w) in high Pi level batch culture (R. E. Beever and D. J. W. Burns 1980). Some

fungi species are reported to accumulate phosphorus in the form of intracellular polyphosphate granules. For instance, *Mucor racemosus* can accumulate poly-P to over 6.7% of its dry weight during active growth (James and Casidale 1964) . Several phosphorus accumulating fungal strains reported in previous studies are summarized in Table 6.

Table 6 reported phosphorus accumulating strain

Species name and reference	Phosphorus composition (% d.w.)	Reference
Mucor racemosus	6.7%	James and Casidale 1964
Mucor ramannianus	6.7%	R. E. Beever and D. J. W. Burns 1980
Mucor Rouxii	7.6%	R. E. Beever and D. J. W. Burns 1980
Cunninghamella echinulata	4.2%	R. E. Beever and D. J. W. Burns 1980
Cunninghamella elegans	high P uptake	Lima and Nascimento 2003

Interestingly, all the reported species share one thing: they are under the Order of Mucorales. The phosphorus compositions in these strains are in comparable magnitude of those in PAOs, indicating the feasibility of using these fungal strains in phosphorus removal and recovery from wastewater.

It could be a controversy because filamentous fungi is an agent that is causing foaming problems known as filamentous bulking, which results in poor sludge settlebility and degraded solid removal (Tchobanoglous et al. 2003). However, this is an issue specific to traditional activated sludge process which is a suspended growth system and gravitational settlement is used for removing bacterial flocs and other particles. In other scenarios - for instance in biofilm reactors where microbial growth is attached to supporting surface - filamentous organisms should cause no problem. As discussed earlier, filamentous fungi are inherent composition of trickling filter process and moving bed bioreactors (MBBR) and filamentous bulking is not associated with these types of processes.

Moreover, a new process was recently proposed to take advantage of the filamentous feature of the fungal cells to induce their cell pelletization during the cultivation (Krull et al. 2010; Krull et al. 2013; Xia et al. 2011). In submerged cultures, many filamentous microorganisms tend to aggregate and grow as pellets/granules. These fungal cell pellets are spherical or ellipsoidal masses of hyphae with variable internal structures, ranging from loosely packed hyphae, forming “fluffy” pellets, to tightly packed, compact, dense granules (Xia et al. 2011). This pelletization process provides another possibility for the harvest of P-containing biomass and thus P fertilizer production.

Figure 13 fungal pellets (*Aspergillus Niger*) formed in flask cultures

To summarize, filamentous fungi are prospective biological agents in wastewater treatment and P removal application. Fungal wastewater treatment has many advantages over current processes which primarily employ bacteria, including (1) higher resistance to toxic material (2) recalcitrant compound degradation through extracellular enzymes (3) better cell harvest and byproducts recover through attached growth and fungal pelletization.

2.3 Materials and methods

2.3.1 Inoculums preparation

Mucor Circinelloides (ATCC 1216B) was selected as the model of phosphorus accumulating fungi for further study. A spore suspension was used for inoculation of the flask cultures. To obtain spores, agar plates with the sporulation medium (39 g/L of Potato dextrose agar, Difco^{TM}) were plated out with spores from a frozen stock (stored in 25% glycerol at -70 ℃) and incubated for 7 days at 27 ℃. After growth, 10 mL sterilized

water was added into agar plate to release the aerial mycelium. The number of spores in the suspension was counted by the optical microscope (National, USA).

2.3.2 Cultivation methods

Fungal cell cultivation were carried out in 250 mL Erlenmeyer flasks containing 100 mL medium on a rotary shaker (INNOVA 42R) at 150 rpm at 27±1 ℃. The culture medium was always sterilized before fungal spores were introduced as the inoculation. Triplicates were performed on each culture experiment. The cultivation conditions were the same for all the experiments unless specifically indicated.

2.3.3 Analytical methods

Fungi biomass was harvested from fermentation broth through centrifugation in all lab-scale experiments: Cultures are transferred into 50mL centrifuge tubes after cultivation, then centrifuged at 7000 rpm for 10 min. The supernatant was collected for COD/nitrogen/phosphorus analysis and precipitant were dried at 105 ℃ overnight for biomass dry weight measurement.

Chemical Oxygen Demand (COD), total nitrogen(TN) and total phosphorus(TP) in the culture media before and after fungal cell culture were analyzed by colorimetric methods using commercial testing kits (TNTplus™ 822/827/845, HACH USA) and a UV-Vis spectrophotometer (Hach® DR 5000™). Soluble COD and soluble phosphorus were measured using the same protocol except that samples are filtrated with 0.45 μm pore size microfiber filter. Phosphorus removal were measured as the difference of total

phosphorus (PO_4^{3-}P) in the media between and after fungal cultivation. Cellular phosphorus content was estimated through calculation, i.e. dividing the mass of removed phosphorus by fungal biomass production.

Samples that were not handled at the same day were stored in -20 ℃ refrigerator.

2.3.4 Screening of phosphorus accumulating strains

The 57 strains used in P accumulating fungi screening are a collection of Dr. Bo Hu's Bioprocessing Group, which are fungi strains isolated from oilseed crop and its surrounding soil by Yan Yang. All strains were cultivated in 24g/L Potato Dextrose Broth (PDB) plus 10mM KH_2PO_4 which is considered as a high P media. After 5 days of cultivation phosphorus removal efficiency and cellular phosphorus content of these strains were assessed.

2.3.5 Growth curve and polyphosphate staining

The flask cultural medium for making ATCC 1216 B growth curve contained PDB (24g/L) and 10 mM phosphate (KH_2PO_4), which provides a phosphorus abundant environment. Total phosphorus in media broth supernatant and the biomass dry weight was measured on daily basis for 7 days. *M. circinelloides* biomass cultivated in PDB (24g/L) with and without 10mM KH_2PO_4 additions are harvested on the 5^{th} of inoculation.

To detect and visualize polyphosphate stored in fungal cells, hyphae was stained following Neisser's method (Serafim et al. 2002) as described below:

Solution preparation:

Solution 1:Part A: 0.1 g Methylene Blue, 5 mL 95% ethanol, 100 mL distilled water. Part B: Crystal Violet (10% w/v in 95% Ethanol) 3.3 mL, 6.7 mL 95% ethanol, 100 mL distilled water. Mix 2 parts of volume of Part A and 1 part volume Part B: Prepare fresh monthly. Solution 2: Bismark Brown (1% w/v aqueous) 33.3 mL, distilled water 66.7 mL.

Procedure: 1. Prepare thin smears on the microscope slides and thoroughly air dry. 2. Stain 30 seconds with Solution 1; rinse 10 seconds with water 3. Stain 1 minute with Solution 2; rinse well with water; blot dry 4. Examine under oil immersion at 1000x magnification with direct illumination. Blue-violet to black is positive (either entire cell or intracellular granules); yellow-brown is negative

2.3.6 Effect of media composition on phosphorus removal of *Mucor circinelloides*

M. circinelloides was studied in batch experiments where the culture media consists of different common carbon, nitrogen and phosphorus sources that may be present in waste streams (Table 7). All media were prepared in the way that the final concentrations of COD, total nitrogen and total phosphorus were approximately 20g/L, 1g/L and 0.5g/L respectively. In addition to carbon, nitrogen and phosphorus, yeast extract 0.2g/L, $MgSO_4 \cdot 7H_2O$ 0.15g/L, KCl 1g/L, $FeSO_4 \cdot 7H_2O$ 0.05g/L and $CaCl \cdot 2H_2O$ 0.05g/L were also added.

Table 7 Media design using different C,N,P sources

	C source	N source	P source
carbon source as variable	glucose	NH_4Cl	KH_2PO_4
	sucrose	NH_4Cl	KH_2PO_4
	starch	NH_4Cl	KH_2PO_4
	fructose	NH_4Cl	KH_2PO_4
	sodium acetate	NH_4Cl	KH_2PO_4
	glycerol	NH_4Cl	KH_2PO_4
	methanol	NH_4Cl	KH_2PO_4
	ethanol	NH_4Cl	KH_2PO_4
nitrogen source as variable	glucose	NH_4Cl	KH_2PO_4
	glucose	$NaNO_3$	KH2PO4
	glucose	urea	KH_2PO_4
	glucose	peptone	KH_2PO_4
phosphorus source as variable	glucose	NH_4Cl	KH_2PO_4
	glucose	NH_4Cl	glycerol phosphate sodium salt

Formula of high strength (COD~5000 ppm) synthetic wastewater: sodium acetate 2g/L, glucose 2g/L, starch 0.5g/L, peptone 0.5g/L, yeast extract 0.25g/L, NH_4Cl 1.2g/L, $MgSO_4 \cdot 7H_2O$ 0.15g/L, $FeSO_4 \cdot 7H_2O$ 5mg/L, $CaCl \cdot 2H_2O$ 5mg/L. Low strength (COD~500 ppm) is made from high strength synthetic wastewater by diluting 10 times with distilled water. KH_2PO_4 was added in the last step to make P:COD ratios at 1:100, 2:100, 4:100 and 6:100 for both high/low strength synthetic wastewater.

2.3.7 Phosphorus removal from real waste streams

Wastewater centrate is the liquid effluent from activated sludge dewatering process. Sample was taken from Metro Council WWTP, MN. Dairy manure was sampled from Jer-Lindy Farm, Brooten, MN. The manure was processed at the farm where large solids were removed by pressing raw manure through a drum screen filter (pore size 0.2 mm). Digested dairy manure was the effluent collected from two 1.2 L lab-scale Upflow Anaerobic Sludge Blanket (UASB) reactors which were fed with dairy manure at HRT of 20 days.

M. circinelloides biomass harvested from 5-day flask culture of 24g/L PDB was rinsed and dispersed in distilled water resulting in 19.6±0.4g d.w./L seeding solution. Flasks loaded with 100mL centrate or screened manure were inoculated with 1 mL seeding solution before incubation. The media were not sterilized, for in real application sterilizing large quantity of waste water is not economically feasible. To account for indigenous microbial activity, experiment controls that are not inoculated with seeding solution were set up for both wastewater centrate and screened manure. On the 5^{th} day the culture in each flask were harvested through centrifugation and analysis was performed for phosphorus removal evaluation.

2.3.8 Statistical analysis

Analysis of variance (ANOVA) was used to test the significance of differences between two or more groups of experimental results. Significant differences were reported at α of 0.05.

2.4 Results and discussion

2.4.1 P accumulating strain screening

Phosphorus removal efficiencies and cellular P content profile of 57 strains are shown in Figure 14. Since the phosphorus concentration in culture media was set at high level for screening purpose, the cellular P contents of most strains excess 1% in this batch experiment. 9 strains including *M. circinelloides* stood out in terms of P removal and/or cellular P content (Table 8). Gene extraction and sequencing result (unpublished data, Yan Yang and Mi Yan) shows 3 of them belong to the Genus *Fusarium*. A *Nigrospora*. An *Alternaria* strain, a *Tremetes* strain and another *Mucor* strain different than *Mucor Circinelloides* are also identified to have extraordinary ability to over-uptake phosphorus.

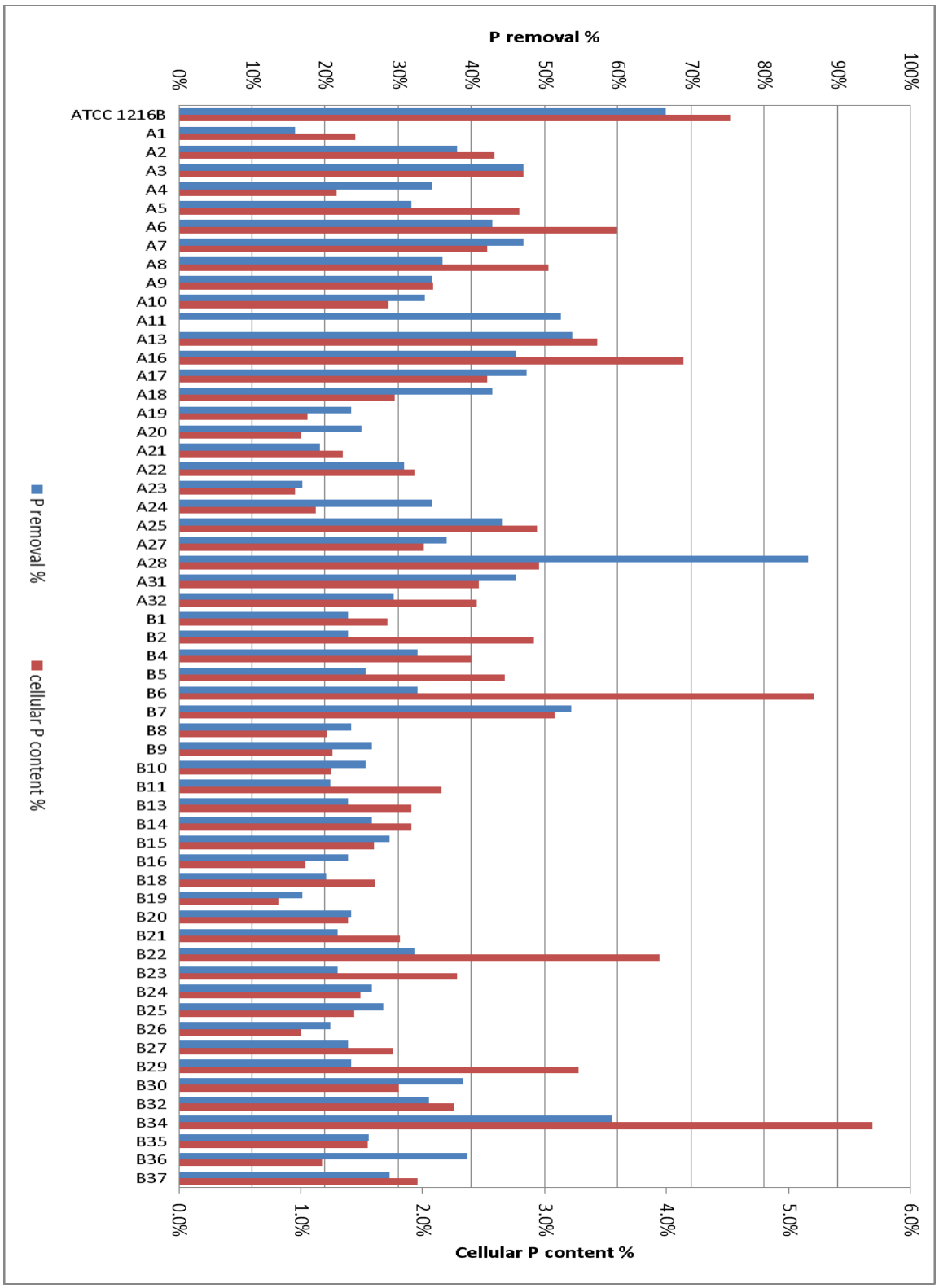


Figure 14 Phosphorus removal efficiencies and cellular P content profile of 57 strains

Table 8 Summary of best performing strains in the screening process

Strain name	P removal (mg/L)	P removal efficiency %	cell P content % d.w.
Mucor Circinelloides	277	67%	4.5%
Fusarium Acuminatum	178	43%	3.6%
Fusarium equiseti	217.4	52%	NA
Fusarium Lacertarum	223.6	54%	3.4%
Nigrospora oryzae	192	46%	4.1%
Alternaria sp.	357.4	86%	3.0%
Trametes pubecens	136	33%	5.2%
Mucor hiemalis	223.2	54%	3.1%

ATCC 1216B *(Mucor Circinelloides)* was chosen as model organism in this study not only because of its good performance in this test and its high growth rate, but also this is an ATCC strain which has been identified as Biosafety Level 1 (BSL-1), having minimum healthy risk for researchers. All the other strains showing their P removal & accumulation potential in this screening process have high value for further study.

2.4.2 Phosphorus removal pattern of *Mucor Circinelloides*

As shown in Figure 15, the exponential growth phase of *M. Circinelloides* was observed between Day 1 and Day 3. Phosphorus concentration in fermentation broth declined in the same period, and kept nearly constant since Day 5. The calculated cellular phosphate is around 5.7-6.0% since Day 2 (the value, 12% on Day 1, was not reliable due to

relative large error measured on very limited amount of biomass), indicating that cell growth, phosphorus uptake and storage are roughly synchronized.

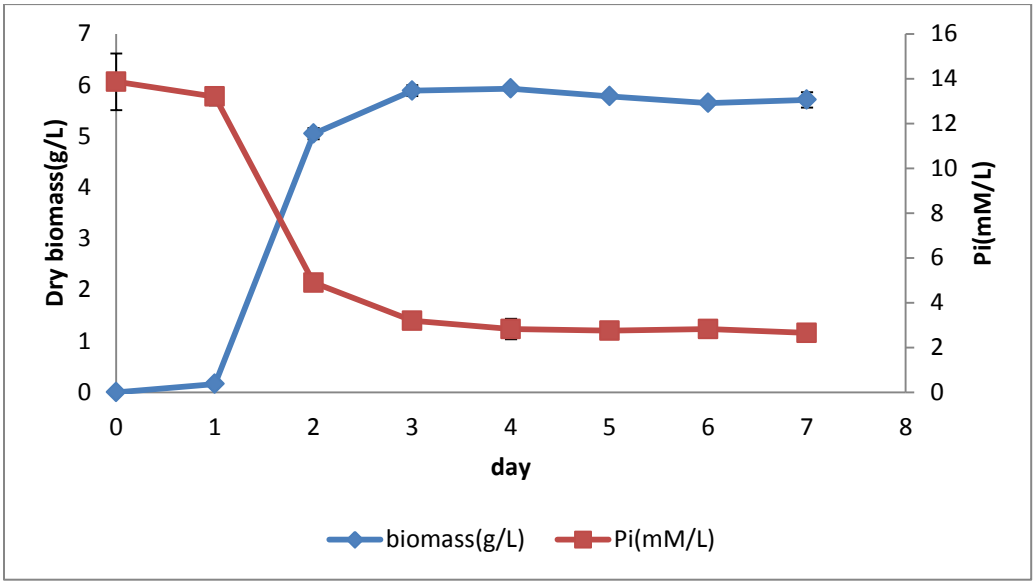
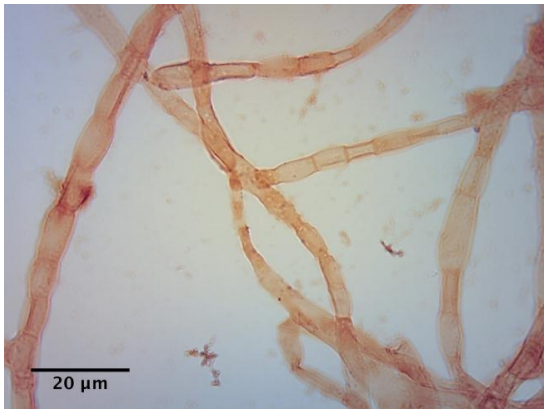


Figure 15 Growth curve of ATCC 1216B in 24g/L PDB with 10mM KH_2PO_4

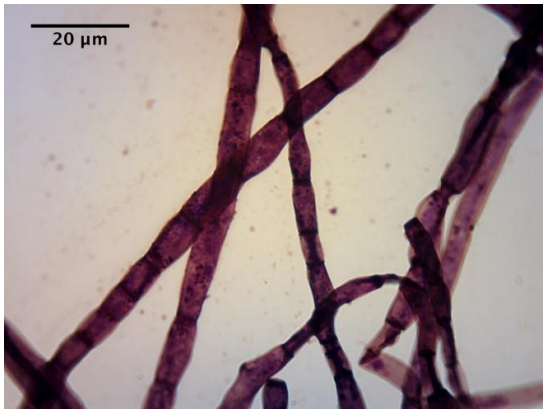
The P uptake pattern of *M. Circinelloides* is different than PAOs present in wastewater treatment plant sludge in that common PAOs need anaerobic/aerobic cycles to complete phosphorus removal, while this fungal strain can continuously uptake phosphate from media during its growth and maintain high cellular phosphorus content (~6%) under a consistent aeration condition (flask culture on 150 rpm shaker in this study). This feature gives fungal P removal a potential advantage over conventional EBPR process that it does not require two separated reactors each has a different aeration configuration, which brings merits like reduced cost and simplified operation.

Figure 16 shows the microscopic structure of fungal hyphae after its growth in 24g/L PDB with or without extra phosphate addition. Without dyeing the cells with Methylene Blue, the fungal hyphae look yellow or brown as can be seen in Figure 16 (A). This is the color of Bismarck brown, the dye for counterstain. The cells would also be yellow or brown if Methylene Blue is applied but there is no polyphosphate exists in specimen, which is considered as Neisser negative. If certain structure of a cell or some granules in the cell has a deep purple to black color, it is considered as Neisser positive and the organism is identified as PAO. In Figure 16 (B) and (C) the entire hyphae were dyed purple, which is interpreted as the storage of large amount of poly-P (Serafim et al. 2002). When *M. circinelloides* grew in 24g/L PDB with extra amount (0.6g/L) of P which makes the P:COD ratio approximately 3:100, the entire hyphae have darker color (Figure 16 (B)) than the media with 24g/L PDB only (P:COD ~ 0.5:100). It implies that the quantity of polyphosphate stored in *Mucor* cells increased with higher environmental P concentration.



A

B



C

Figure 16 Neisser staining of *M. circinelloides*

(A) *M. circinelloides* hypha with counterstain (Bismarck brown) only (B) Stained hypha of *M. circinelloides* Culture media: 24g/L PDB. (C) Stained hypha of *M. circinelloides*. Culture media: 24g/L PDB with additional phosphate (0.6g/L)

2.4.3 Effect of media composition on phosphorus removal of *Mucor circinelloides*

Mono sugars like glucose and fructose correspond to highest phosphate removal when they are used as single source of carbon (yeast extract used in low concentration as supplement also contributes to carbon, however its small amount compared to other major substrates would not change the nature of this study). Sucrose does not facilitate *M. circinelloides* to uptake phosphorus very well. This is consistent with previous observation that phosphorus accumulation is directly related to the biomass growth and *Mucor Circinelloides* has poor sucrose utilization for growing cells (Data not published yet). Starch is comparative to mono sugars, probably due to the high-efficiency Amylase of *Mucor*. All these carbohydrate are common components that can be found in household waste. However most of these easy sugars will be consumed by bacteria in the wastewater sewer or in holding tank. Acetate and alcohols are the degradation products of anaerobic process, which can be found in wastewater after long-time transportation or storage. Acetate turned out to be a good carbon source for P removal, while P removal is significantly lower when *M. circinelloides* was feeding on methanol and ethanol.

For nitrogen, organic N source shows obvious advantage over inorganic compounds, i.e. ammonium and nitrate. Possible explain is that peptone does not only serve as organic nitrogen source, it also provides a variety of other nutrients for cell growth. In municipal wastewater treatment process, ammonium-nitrogen and organic nitrogen (or TKN, the combination of two) are the major forms of nitrogen in influent wastewater, and a typical ratio of ammonium-N to organic nitrogen is 1:2 (van Haandel and van der Lubbe 2012). The results of this experiment indicate that fungal treatment process should be placed in

early stage where organic nitrogen is prevalent, if it is aimed for phosphorus removal & recovery.

The form of phosphorus also has significant impact on its availability to fungi. Figure 8 (C) shows that inorganic phosphate was subjected to higher removal compared with its organic counterpart. This means to achieve efficient conversion of phosphorus from agricultural wastewater like dairy manure to fungi, it is important to release soluble, inorganic from particulate, organic phosphorus which contribute to large portion of phosphorus in this type of wastewater.

Batch experiment using synthetic wastewater show that *M. circinelloides* can remove ~ 72-82% P when P to COD ratio is roughly 1:100 (Figure 18 A and B), which is similar to the wastewater that carry low concentration of phosphate. Noting that this removal efficiency is generated from simple flask culture process, potentially higher removal efficiency or even complete removal can be expected after it applied to continuous process and the process parameters such as DO, F:M , HRT and SRT are optimized. This result also reveals that by fixing COD and raising P concentration only, net P removal increases, but it is not proportional to P:COD ratio.

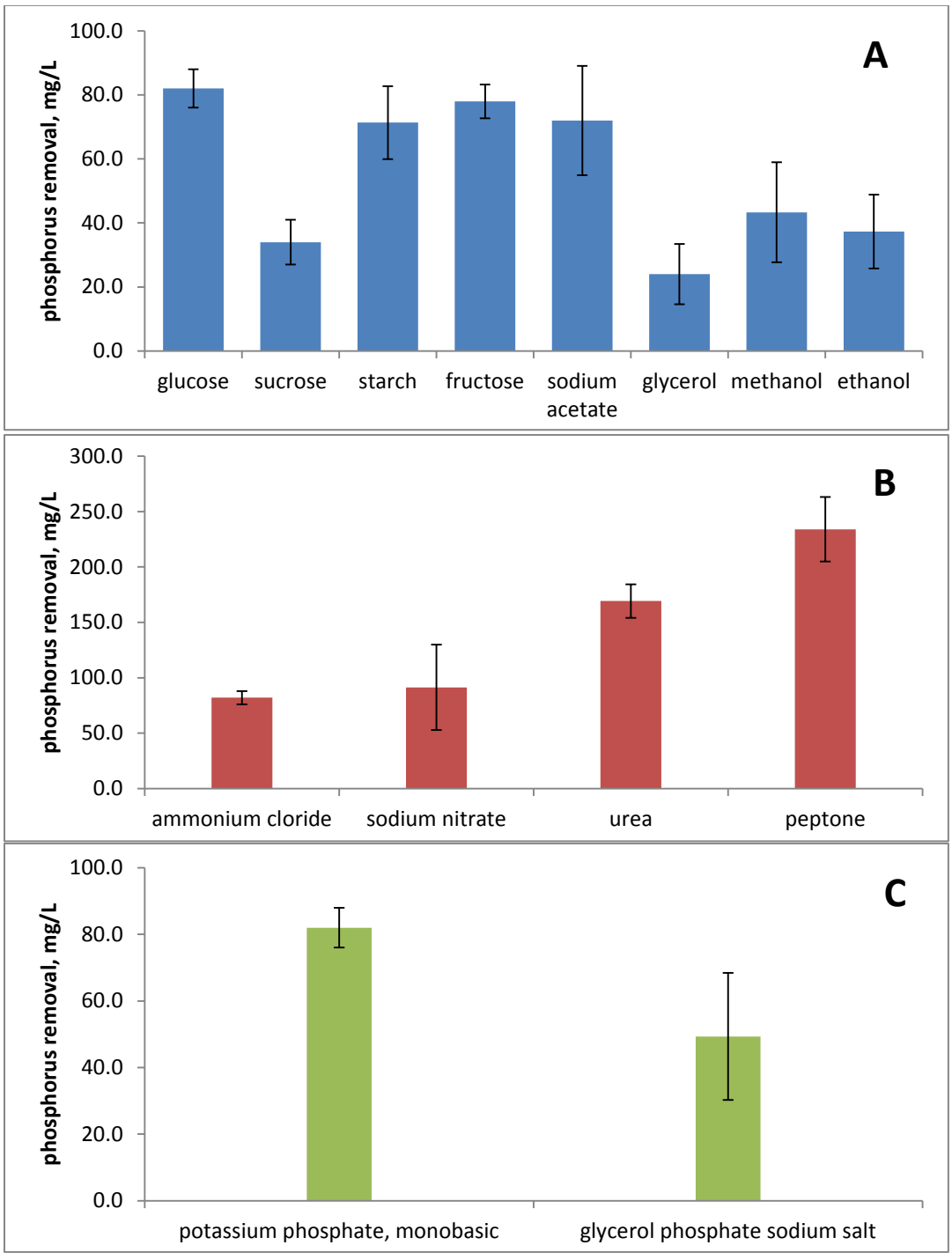


Figure 17 phosphorus removal by *Mucor Circinelloides* when different carbon (A), nitrogen(B), and phosphorus (C) sources are utilized

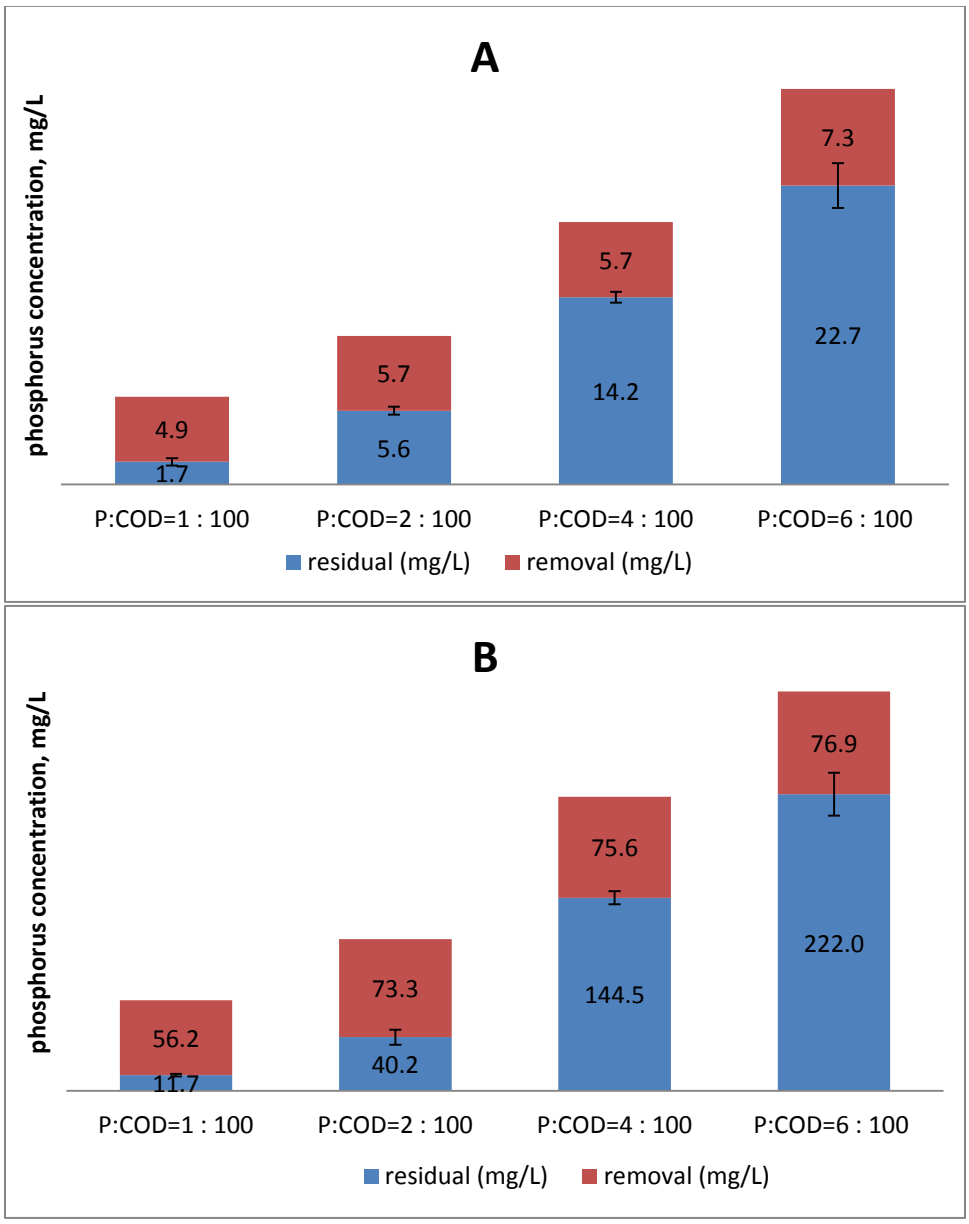


Figure 18 Phosphorus removal by *Mucor Circinelloides* using synthetic wastewater of low strength (A) and high strength (B) with different P:COD ratio

2.4.4 Phosphorus removal from real waste streams

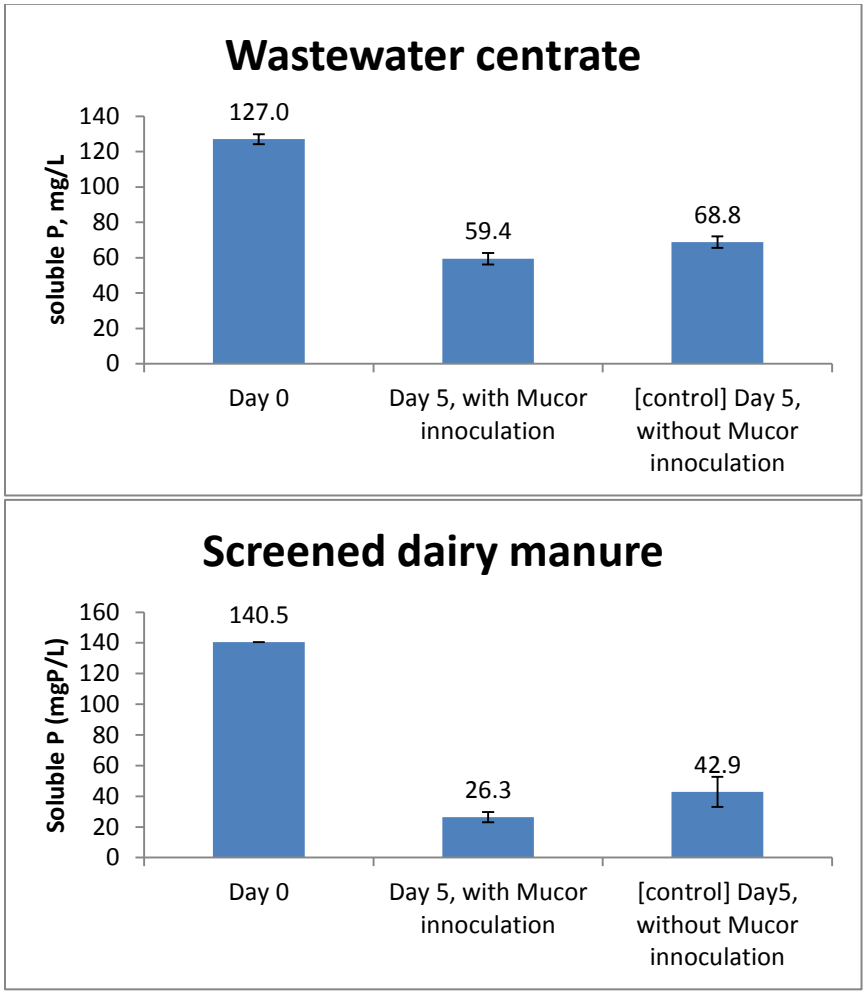
Figure 19 shows the initial & Day 5 soluble P concentrations in wastewater centrate, screened dairy manure and digested. All waste streams contain high concentration of

soluble phosphorus, i.e. 127 mg/L, 140.5 mg/L and 23.4 mg/L respectively. Theoretically, anaerobic digestion removes only a small portion of phosphorus through biomass assimilation. However, as the manure and digested manure used in this study were derived from different batch of samples and have different degrees of dilution, the significant difference in manure and digested manure P concentrations was anticipated.

After 5 days' flask culture, the soluble P concentration in wastewater centrate with *M. circinelloides* dropped to 59.4±3.24 mg/L. The experiment control which did not have *Mucor* inoculation also showed a reduction in soluble P, with a final concentration of 68.8 ±3.34 mg/L. This removal can be explained by the growth and phosphorus uptake by indigenous bacteria.

High Phosphorus removal baselines in experiment controls were also observed in both screened dairy manure and digested manure. For dairy manure, Day 5 soluble P concentration was 26.3 ±3.5 mg/L with *Mucor* inoculation and 42.9 ±9.8 mg/L in experiment control. For digested manure, Day 5 soluble P concentration was 9.6 ±0.5 mg/L with *Mucor* inoculation and 10.7 ±1.1 mg/L in experiment control. Statistical analysis shows that the differences in Day 5 soluble P concentration between treated sample (with *Mucor* inoculation) and experiment control (without *Mucor* inoculation) were significant when wastewater centrate ($p=0.025$) and screened manure ($p=0.050$) was used as media, whereas it was not significant for digested manure ($p=0.211$). Thus the difference in P removal can be explained by the inoculation of *M. circinelloides.* However, the P removal baselines were so high that the groups treated with fungi merely

show minor improvements in terms of P removal efficiency (16.1%, 19.7% and 0.9% for wastewater centrate, dairy manure and undigested respectively) compared to experiment controls. The reasons could be (i). the growth of fungi was limited by the competition with indigenous microbial consortia in the waste streams (ii). Process parameters such as F:M/SRT/HRT were not controlled in flask cultures.



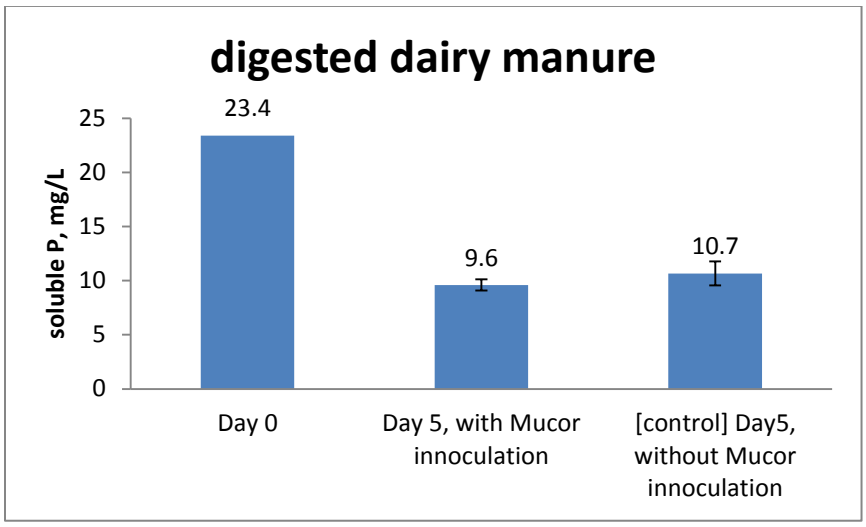


Figure 19 Phosphorus removal from real waste streams on Day 5

2.5 Conclusions and future work

This study proposed an innovative process for wastewater phosphorus removal and recovery using phosphorus accumulating fungi. 9 strains are identified to possess high phosphorus removal and storage potential of which *M. circinelloides* was studied of its feasibility in wastewater treatment application. The merits of applying fungi in wastewater treatment include

- High resistance to toxic material
- Recalcitrant compound degradation through extracellular enzymes
- Cellular P composition of some strains can reach about 7% which is comparable to PAOs
- Continuous high-rate P uptake process
- Easiness of harvest due to the fungal pelletization technology

To fully explore the potential of using filamentous fungi in wastewater P removal and recovery, a continuous pilot reactor will be set up and real wastewater will be used as feeding material in future research. A study on cost-efficient phosphorus release process for manure and other agricultural waste stream is under-going and it will facilitate fungal phosphorus recovery. Other proposed researches include process development for pelletization and other harvest technology, and the field test for the validation of fungal phosphorus fertilizer.

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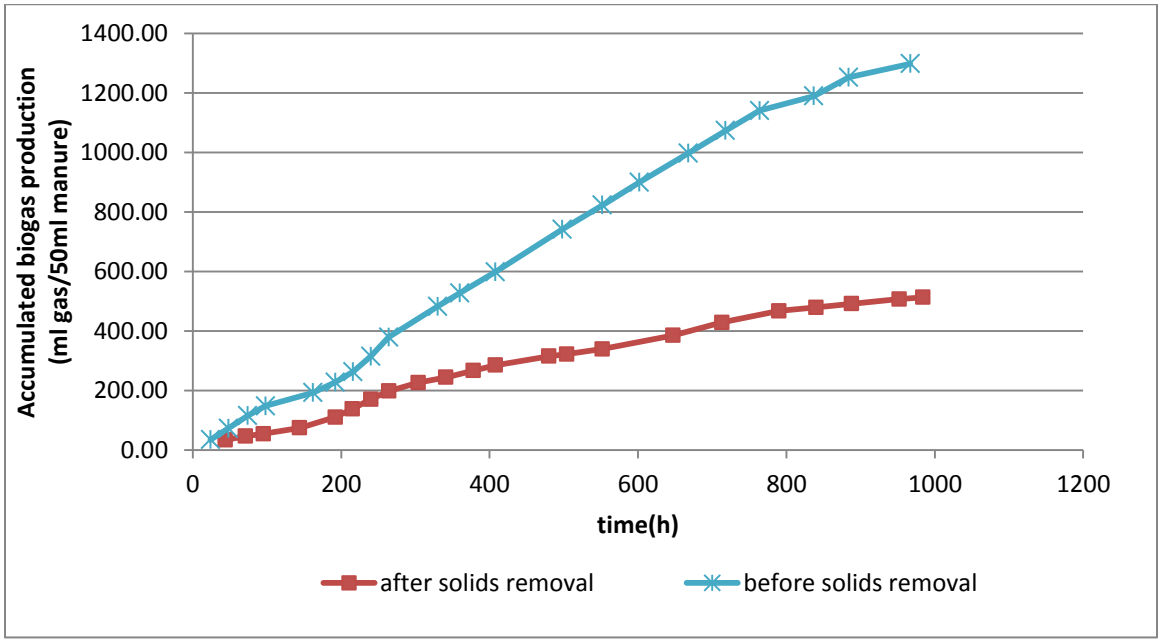
Appendices

Impact of dairy manure solids removal

After solids were removed from the dairy manure, TS and COD of the manure were nearly halved. Batch experiment showed that biogas production (volume basis) decreased by 60% as a result of solids removal.

	TS(mg/L)	COD(mg/L)	TN(mg/L)	TP(mg/L)
Before solids removal	78004±3162.09	92433.33±19656.13	2623.33±156.31	702.33±41.14
After solids removal	37845.33±224.58	45700±7534.59	2693.33±690.6	562±7.55



BMP tests without dilution and inoculation

In a preliminary experiment kitchen waste was mixed with 50 mL dairy manure and sealed in serum bottle. No dilution and inoculation were used during this test. After 100 hours, the gas production almost ceased in the treatments with kitchen waste added to the manure. It is speculated that the process failure was caused by organic overloading and insufficient inoculation, as pH drop was observed for the treatments with kitchen waste added. It was concluded that sufficient inoculation is important to BMP tests.